

"On the Culture of the Nitroso-bacterium." By H. S. FREMLIN, Lymph Laboratories, Chelsea Bridge. Communicated by Sir MICHAEL FOSTER, K.C.B., Sec. R.S. Received February 23,—
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(Carried on at Westminster Hospital Medical School and the Jenner Institute of Preventive Medicine.)

My object in undertaking this work was in the first place to obtain a pure culture of the nitroso-bacterium; in the second place to discover whether it really was a fact that this species was unable to grow in the presence of organic matter, as stated by Winogradsky.*

My experiments with the nitroso-bacterium appear to show that:—

1. A practically pure culture of the bacterium can be obtained after sub-culturing for 7 months in Winogradsky's ammonia solution.
2. That the nitroso-bacterium will grow in this solution in the presence of organic matter.
3. That the nitroso-bacterium will grow not only on silica jelly, but also in any ordinary organic medium.

In the course of these experiments pure cultures were again and again obtained by plate cultivation from a great variety of artificial media. Single colonies therefrom were sub-cultured, and these were commonly competent to convert ammonia in a solution into nitrous acid. I infer, therefore, that there are not two separate and distinct species of bacterium, morphologically similar, and able to persist side by side indefinitely in inorganic solutions apart altogether from other bacteria, the one able to convert ammonia into nitrous acid and cultivable only in special media, the other growing on ordinary media but with no ability to convert ammonia into nitrous acid.

The purpose of my paper is to show that I have been dealing with a single species of bacterium, which is not only able to oxidise ammonia but is capable also of growing in ordinary organic media.

The solution used by Winogradsky for growing the nitroso-bacterium consists of water containing 1 per 1000 ammonium sulphate, 1 per 1000 potassium phosphate, and 1 per 100 magnesium carbonate. The carbonate solution is sterilised separately, and added to the solution of salts after sterilising, to prevent chemical decomposition.

This solution I have continued to use all through the work, and in this paper it will be referred to as the "ammonia solution." It has

* It was in 1895 that, in view of Winogradsky's work, I commenced these investigations. Since that date, and while my own research has been in progress, I have studied, in their bearing on the subject of my labours, a number of papers in various journals by a number of observers, whether in criticism or in support of Winogradsky's thesis, as will be seen on reference to my "full" paper.

always been tested for the presence of oxides of nitrogen, and a control set up when batches of the solution were inoculated.

The presence of nitrites was judged by a solution of diphenylamine in sulphuric acid, Ilosvay's solution being used as a control when necessary.

When I commenced work I obtained cultures of the nitroso-bacterium by inoculating ammonia solutions with small quantities, 0.2 gramme or less, of various kinds of soil; rich garden soil, humus, sand, &c. Tubes of ammonia solution so inoculated were kept at room temperature and placed in a dark cupboard in order to avoid exposure to light.

The evidence of the growth of the nitroso-bacteria was found in the conversion of the ammonia in the solution into nitrous acid. This is at first a slow process and does not commence in the tubes for some 3 weeks as a rule; usually it requires another week or two to be completed.

I inoculated ammonia tubes with soil 43 times. Of these 70 per cent. showed oxidation of the ammonia.

Gelatine plates poured from these tubes showed moulds, yeasts, liquefying and non-liquefying bacteria, and also a micro-organism morphologically similar to the nitroso-bacterium.

1st Dilutions.—From the ammonia tubes which showed oxidation sub-cultures were made in like media. Of these 77 per cent. showed nitrites. This occurred in 8 weeks.

2nd Dilutions.—From the first dilutions sub-cultures were made. Of these 85 per cent. showed oxidation in from 2 to 3 months.

3rd Dilutions.—From the second dilutions sub-cultures were made. All these showed formation of nitrite in one month.

4th Dilutions.—Sub-cultures from the third dilutions exhibited formation of nitrite in 93 per cent. of the tubes inoculated. These, as a rule, required 6 weeks before this was completed.

5th Dilutions.—Sub-cultures were made from the fourth dilution tubes. Of these 77 per cent. showed the formation of nitrite; the time required being 2 months.

With regard to the microscopical specimens made from the several tubes; the 1st, 2nd, and 3rd dilutions showed, associated with the nitroso-bacterium, rod-shaped micro-organisms, in gradually diminishing numbers in succeeding dilutions. The 4th and 5th dilutions gave almost pure culture of the nitroso-bacterium apparently.

Ammonia Solution containing no Carbonate.

A medium containing simply ammonium sulphate and potassium phosphate was tried.

The nitroso-bacterium was able to grow in this, and to produce nitrite; but the micro-organism did not develop in sub-cultures.

Liquid Media containing Organic Matter.

I made a series of experiments with "ammonia solutions" containing peptone beef broth, Witte's powdered peptone, and urea.

Beef Broth.

This was added in quantities varying from 1 in 11000 to 10 in 100.

Cultures of nitroso-bacteria grew well when inoculated from inorganic solutions into the lower percentages of beef broth, and on transferring them to higher percentages they were able to continue their nitrification. If cultures of nitroso-bacteria were taken from inorganic solutions and placed directly into solutions containing beef broth to the extent of 1 in 1000 they failed to show oxidation.

Peptone.

This was used in solutions containing 1 in 11000 and 1 in 5000.

The nitroso-bacterium was able to grow in these solutions; its development being satisfactory, as shown by the formation of nitrite.

Urea.

"Ammonia solutions" were prepared which contained from 1 in 11000 to 1 in 1000 of urea.

The nitroso-bacterium although developing in the presence of small quantities of urea failed to do so when the solution contained as much as 1 in 1000.

The above experiments show that the nitroso-bacteria can grow in the presence of organic matter. The addition of small quantities of organic matter to ammonia solution containing the nitroso-bacterium does not apparently check the formation of the nitrous acid.

The addition of larger percentages of organic matter to "ammonia solutions" does tend to check and finally stop the action of these bacteria if they are introduced directly from soil, or from inorganic media.

The experiments also show that, where nitroso-bacteria have oxidised ammonia in solutions which contain small quantities of organic matter, they are able to continue this work when transferred to solutions of ammonia containing amounts of organic matter that entirely arrest their oxidising action when they are transferred thereto direct from inorganic solutions.

*Isolation of the Nitroso-bacterium.**Plate Cultures.*

In carrying out the work of isolation of the nitroso-bacterium I made plate cultures containing silica, gelatine, and agar media respectively.

Silica Plates.

I found that numerous species of micro-organisms grew on this medium, and that, therefore, it was necessary to use what seemed to be pure cultures of the nitroso-bacterium, as this species does not form colonies rapidly, and is liable to be smothered by the more quickly-growing bacteria if one attempts to isolate the nitroso-bacterium from soil or a very impure culture. The nitroso-bacterium grows well in this medium, and in one instance I was able to remove a single colony which oxidised the ammonia in a solution. From this culture inoculation was made into beef-broth agar, and plates poured. These plates grew large numbers of colonies in pure cultures.

Numerous colonies were taken from silica plates and grown on beef-broth agar, but such growth transferred to ammonia solutions did not produce nitrification. This being the case I thought that probably the micro-organism had lost its power of oxidising the ammonia, so that I tried to devise a means by which this function might be re-established; and for this purpose the micro-organism was placed in as natural surroundings as were attainable. The following was the method adopted. A single colony was taken from silica plate and inoculated on to sloping beef-broth agar. After growing there it was transferred to a sterile ammonia solution; this was allowed to filter daily through sterile soil, thus allowing of aeration of the growth whilst in its natural surroundings. This experiment succeeded, nitrite being formed in 10 weeks. A control filter showed no change.

Gelatine Plates.

Winogradsky states that the nitroso-bacterium does not grow on gelatine; so that, in the first place, the method that he advocates to obtain a pure culture of nitroso-bacteria was adopted.

Particles of magnesia were removed from an oxidised ammonia solution and sown on to gelatine plates. Now if these particles carried nitroso-bacteria alone there would, if Winogradsky be correct, be no growth, and such a particle showing no growth could be removed and reinoculated into an ammonia solution, and thus a pure culture obtained. But I found that around particles so inoculated into gelatine, colonies invariably occurred. It was noted that these colonies were in pure culture and were made up of an oval organism that was morphologically similar to the nitroso-bacterium.

Secondly, gelatine plates were poured from oxidised ammonia solutions; these also gave the same species of micro-organism, often in practically pure culture. From one such plate a piece was removed, and in an ammonia solution it produced oxidation. This oxidised ammonia solution again yielded the same species on gelatine plates.

Gelatine media prepared from divers soils and inoculated with oxidised ammonia solutions exhibited the same species of micro-organism.

Hence it is to be inferred that, either the nitroso-bacterium grows on gelatine, or that an organism morphologically similar occurs in the same inorganic solutions with the nitroso-bacterium and thrives like it in inorganic solutions.

To arrive at some definite conclusion on this point further experiments were made with agar plates.

Agar Plates.

In commencing my researches with Agar, beef-broth agar was used.

Beef-broth Agar.

Plates of this medium, inoculated from cultures containing nitroso-bacteria gave similar results to those with gelatine; that is to say, micro-organisms, morphologically similar to the nitroso-bacterium, grew well on agar, as they had done on gelatine.

Pieces of these plates showing colonies were on fifty-three occasions inoculated into ammonia solutions; of these fifty-three solutions twenty showed formation of nitrite.

Pieces of such plates, on which no colonies were found, were in nineteen instances inoculated into ammonia solutions. In no case did the formation of nitrite occur.

Hence we have :—

Agar Plates with Colonies.

Inoculated.	Oxidised.
53	20

Agar Plates without Colonies.

Inoculated.	Oxidised.
19	0

I also made numerous experiments with a medium which I term “ammonia agar.” This consists of :—

Ammonium sulphate, 1 gramme.
Potassium phosphate, 1 gramme.
Distilled water, 1 litre.

The salts are dissolved, and agar added to $1\frac{1}{2}$ per cent. ; the whole being boiled up and prepared as ordinary agar. Magnesium carbonate is added after sterilisation.

As will be seen, this agar corresponds in composition to the ammonia solution used for the ordinary cultures, save for the presence of the $1\frac{1}{2}$ per cent. agar. It has a slightly lower melting and coagulation point than bouillon agar.

I have poured over 100 plates of this medium. It grows the nitroso-bacterium well, oxidation of the ammonia in the plate occurring within 2 months, as a rule, after inoculation with oxidised ammonia solutions.

All plates that showed oxidation of the ammonia contained large numbers of colonies of apparently the same bacterium. This organism being oval in form, and associated with the formation of nitrite, and being often almost or altogether in pure culture, must be considered to be the nitroso-bacterium. It occurred in the dilution plates in all instances. Nevertheless but few of these showed oxidation of the ammonia.

The following table gives results obtained :—

	Plates, number poured.	Number showing oxidation.
Original	26	22
First dilution.....	26	3
Second dilution.....	26	1

This shows that unless the colonies were numerous nitrite was not formed.

In one instance a single colony taken from an ammonia agar plate and placed on sloping ammonia agar formed nitrite in 9 months. Plates of beef-broth agar and gelatine poured from this culture grew enormous numbers of the colonies. These colonies develop both at room temperature and 37° C. At room temperature, after 6 days, the colonies appear to the naked eye as white iridescent growths varying in size. Some days later they become lemon coloured, and finally yellow. Under the microscope they were seen to be made of micro-organisms corresponding to the nitroso-bacteria.
